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N-(2-MERCAPTOETHYL)-1,3-PROPANEDIAMINE (WR-1065) PROTECTS THYMOCYTES FROM PROGRAMED CELL DEATH[1]

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Gamma-irradiation, glucocorticoid hormones, and calcium ionophores stimulate a suicide process in thymocytes, known as apoptosis or programed cell death, that involves internucleosomal DNA fragmentation by a Ca^{2+}- and Mg^{2+}-dependent nuclear endonuclease. In this study we report that *N*-(2-mercaptoethyl)-1,3-propanediamine (WR-1065) blocked DNA fragmentation and cell death in thymocytes exposed to γ-radiation, dexamethazone, or calcium ionophore A23187. WR-1065 protected the thymocytes from radiation-induced apoptosis when incubated with cells after irradiation but not before and/or during irradiation. WR-1065 inhibited Ca^{2+}-and Mg^{2+}-dependent DNA fragmentation in isolated thymocyte nuclei. Our results suggest that WR-1065 protects thymocytes from apoptosis by inhibiting Ca^{2+}- and Mg^{2+}-dependent nuclear endonuclease action.

Thymic small lymphocytes, commonly known as thymocytes, undergo a suicide process known as apoptosis or programed cell death in response to several stimuli, including exposure to γ-radiation (1–3), glucocorticoid hormones (4–6), calcium ionophores (6), antibodies to the CD3-TCR complex (7), or the environmental contaminant 2,3,7,8-tetrachlorodibenzo-γ-dioxin (8). The apoptotic death of thymocytes, lymphocytes, and intestinal crypt cells after clinically relevant doses of irradiation (2–5 Gy[3]) distinguishes them from most other cells, which undergo reproductive death at these radiation doses (9–14). In reproductive death the cell functions until it attempts one or more cell divisions, after which it dies (15). In apoptosis, however, the damage manifests itself in the absence of mitosis. Apoptosis is characterized by several morphologic and biochemical changes, including plasma and nuclear membrane blebbing, impairment in membrane permeability, chromatin condensation, DNA fragmentation, and impairment of ATP synthesis (9). The most characteristic biochemical marker for apoptosis is nuclear DNA fragmentation into oligonucleosomal subunits that can be recognized from random cleavage observed in cells undergoing necrosis (1–6).

The radioprotectant drug WR-2721 is a well-known protective agent that selectively protects normal tissues against cytotoxicities of radiation and chemotherapeutic alkylating agents (16–21). WR-1065 the dephosphorylated form of WR-2721 and generally considered to be the active form of the drug, has been shown to protect the mammalian cells in vitro from radiation-induced reproductive death (22–25). In this report we describe the protective effects of WR-1065 on apoptosis in thymocytes induced by γ-radiation, dexamethazone, and calcium ionophore A23187.

MATERIALS AND METHODS

TCM. RPMI 1640 medium supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 55 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% heat-inactivated FCS was used in all the studies.

Cell isolation. CD2F1 male mice, 6 to 7 wk old, were asphyxiated with CO_2, and their thymuses were removed and placed in TCM on ice. Single cell suspensions were prepared by pressing the organs through wire mesh screens followed by passage through a 25-gauge needle. The suspensions were washed once in TCM and resuspended in cold Tris-buffered isotonic ammonium chloride to lyse the red cells (26). The cells were washed once in TCM and resuspended in TCM. Viable cell numbers were determined by trypan blue dye exclusion method (27).

γ-Irradiation. Thymocytes (2×10^6/ml) were exposed to 1.5 to 6.0 Gy ^{60}Co γ-radiation at a dose rate of 1 Gy/min.

Incubation of thymocytes. Immediately after irradiation, cells were centrifuged at 200 × *g* for 10 min, resuspended in fresh medium at 2×10^6 cells/ml, and incubated with WR-1065 in TCM containing 100 U/ml catalase at 37°C in a humidified incubator under an atmosphere of 5% CO_2 in air.

Dexamethazone was dissolved in a minimum volume of ethanol and diluted to the desired concentration with TCM. Thymocytes were incubated with different concentrations of dexamethazone in TCM with or without WR-1065 as described above. A similar quantity of ethanol was added to controls. The studies were repeated with different concentrations of calcium ionophore A23187.

DNA fragmentation assay. At selected times cells were harvested by centrifugation at 200 × *g* for 10 min. The cells were lysed with 0.5 ml ice-cold hypotonic lysing buffer (10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 0.2% Triton X-100) and centrifuged at 13,000 × *g* for 20 min to separate intact from fragmented DNA. The pellet was then sonicated for 10 s in 0.5 ml lysis buffer. DNA in the pellet and supernatant fractions was determined by an automated fluorometric method using Hoechst 33258 fluorochrome (28, 29), modified for our studies. This DNA analysis is based on the ability of Hoechst 33258 to bind DNA quantitatively to form a fluorescent complex.

A stock solution of Hoechst 33258 (1 mg/ml) was prepared in distilled water. This solution is stable for 2 wk if kept at 4°C in the dark. A 1 μg/ml working solution was prepared daily by diluting the stock with running buffer (0.5 M phosphate buffer, pH 7.0, containing 0.05% Brij S-35). The working dye container was wrapped in aluminum foil to protect it from ambient light during the analysis.

Continuous flow analysis was performed with Technicon Autoanalyzer II components (Technicon Instruments Corp., Tarrytown, NY), including an autosampler fitted with a 40-place sample tray, a single

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[3] Abbreviations used in this paper: Gy, gray; TCM, tissue culture medium; WR-1065, *N*-(2-mercaptoethyl)-1,3-propanediamine; WR-2721, S-2-(3-aminopropylamino)-ethylphosphorothioic acid.

speed proportioning pump, and a fluoronephelometer. The fluorescence signal was directed to a Hewlett-Packard 3390A integrator (Hewlett-Packard, Downer's Grove, IL), which automatically identified and quantitated sample peaks. All tubes were flow-rated Tygon tubing (Fisher Scientific, Pittsburg, PA). We used a sampler cam that allowed the analysis of 40 samples/h with a 1 min running buffer wash between 30-s sample draws. A minimum sample volume of 0.35 ml was required with the pump tube used (0.6 ml/min).

The concentration of DNA corresponding to the peak height value of each sample was calculated from a curve of calf thymus DNA standards, by using a computer software. We found that fluorometric autoanalysis of DNA is more sensitive and reproducible than diphenylamine method. Sample concentrations ranging from 1 to 20 μg/ml were easily analyzed with the system. The sensitivity can be increased by increasing the volume of the sample draw and adjusting the sensitivity of the fluoronephelometer and integrator. Measurements were unaffected by the presence of cell homogenates or reagents in the sample.

Percentage of DNA fragmentation refers to the ratio of DNA in the 13000 × *g* supernatant to the total DNA in the pellet and 13000 × *g* supernatant.

DNA electrophoresis. The pellets and the supernatants were incubated with RNase (50 μg/ml) for 1 h at 37°C. After this incubation, 50 μg/ml proteinase K were added and the incubation continued for an additional 1-h period. The DNA was sequentially extracted with equal volumes of phenol and chloroform:isoamylalcohol (24:1). The aqueous phase was precipitated with two volumes of ethanol at -20°C overnight. Pellets were air dried and resuspended in Tris-EDTA buffer (10 mM Tris-HCl, pH 7.8, and 1 mM EDTA). Horizontal electrophoresis of DNA was performed for 2.5 h at 100 V in 0.75% agarose gel with 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0, as running buffer. DNA was visualized after electrophoresis by ethidium bromide staining.

Nuclei isolation and endogenous nuclease activity. Nuclei were prepared from the thymocytes by the method of Cohen and Duke (5). The nuclei were suspended in Tris-buffered (10 mM, pH 7.5) isotonic sodium chloride and incubated at 37°C for 4 h in the presence of different cations. After incubation, the nuclei were sedimented at 200 × *g* for 10 min. The supernatant was discarded because it contained no DNA. The nuclear pellet was lysed with lysis buffer, and intact DNA and fragmented DNA were estimated as described for whole cells.

Materials. RPMI 1640 medium, 2-ME, and the antibiotic mixture were purchased from GIBCO, Grand Island, NY; FCS was obtained from HyClone Laboratories, Logan, UT; Hoechst 33258 fluorochrome was purchased from Calbiochem-Behring, La Jolla, CA; Dexamethazone and calcium ionophore A23187 were purchased from Sigma Chemical Co., St. Louis, MO; and WR-2721 and WR-1065 were kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

RESULTS

WR-1065 inhibits radiation-induced DNA fragmentation and cell death in thymocytes. Single cell suspensions were prepared from thymuses and exposed to different doses of γ-radiation. After irradiation the thymocytes were resuspended in fresh medium containing 10 mM WR-1065 and incubated at 37°C as described in *Materials and Methods*. The level of DNA fragmentation was determined at various times postirradiation. DNA fragmentation increased with radiation dose and with time postirradiation. (Fig. 1, *A-D*). It is interesting to note that DNA fragmentation was completely blocked in cells incubated with WR-1065 after different doses of γ-radiation (Fig. 1, *A-D*). In all experiments, the background DNA fragmentation in unirradiated thymocytes increased with time to a maximum level of 10 to 15% at 8 h. There was no background DNA fragmentation in unirradiated thymocytes after WR-1065 treatment (Fig. 1*A*).

Electrophoretic analysis of pellet and supernatant DNA isolated from 6.0 Gy-irradiated thymocytes showed typical "ladder" pattern, consisting of DNA fragments of a size, multiple of 200 bp unit (Fig 2, *lanes D* and *H*, respectively). This pattern of DNA fragmentation has already been shown after γ-irradiation (1, 2) and glucocorticoid treatment (4) of thymocytes. The pellet DNA

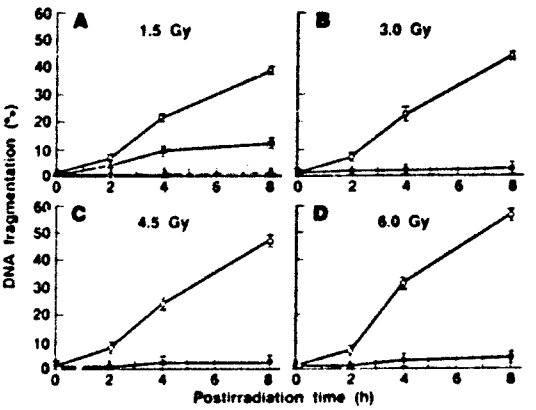

Figure 1. Effect of WR-1065 on DNA fragmentation in thymocytes exposed to increasing doses of γ-radiation. Thymocytes (2 × 10^6) were irradiated in TCM at a dose rate of 1.0 Gy/min. The percentage of DNA fragmentation was measured after various times of incubation with or without 10 mM WR-1065, under the conditions mentioned in *Materials and Methods*. The results are mean ± SE from three experiments. ▲, unirradiated; △, unirradiated + WR-1065; C, irradiated; ●, irradiated + WR-1065.

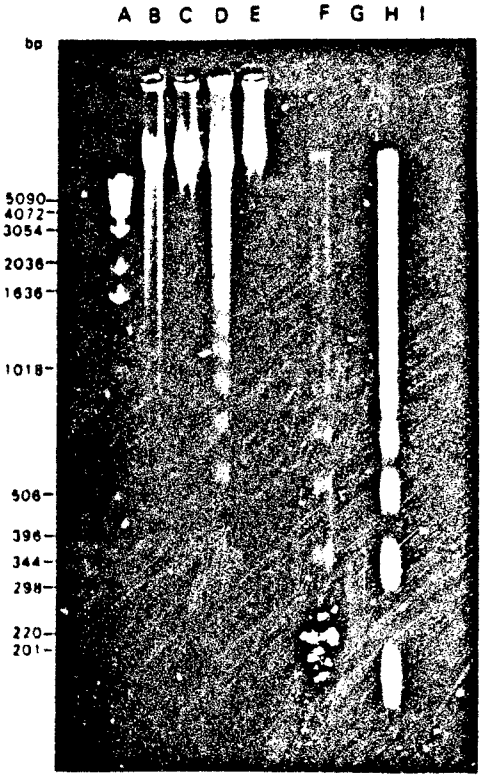

Figure 2. Agarose gel electrophoresis of pellet and supernatant DNA isolated from unirradiated or 6.0 Gy-irradiated thymocytes after 6 h incubation with or without 10 mM WR-1065. The molecular size standard DNA is a 1-kb DNA ladder purchased from GIBCO. *Lane A*, standard 1-kb DNA ladder; *lane B*, unirradiated-pellet; *lane C*, unirradiated + WR-1065-pellet; *lane D*, 6.0 Gy-pellet; *lane E*, 6.0 Gy + WR-1065-pellet; *lane F*, unirradiated-supernatant; *lane G*, unirradiated + WR-1065-supernatant; *lane H*, 6.0 Gy-supernatant; *lane I*, 6.0 Gy + WR-1065-supernatant.

isolated from irradiated thymocytes after WR-1065 treatment was of high m.w. and remained at the top of the gel, and there was no "ladder" pattern of DNA bands (*lane E*). The supernatant obtained from irradiated-thymocytes treated with WR-1065 had no DNA fragments (*lane I*). The pellet and supernatant DNA of unirradiated thymocytes contained a small amount of fragmented DNA

(*lane B* and *lane F*, respectively). The pellet DNA isolated from unirradiated cells treated with WR-1065 was of high m.w. (*lane C*). There were no DNA fragments in the supernatant isolated from unirradiated cells treated with WR-1065 (*lane G*). These results clearly indicate that WR-1065 protects the thymocytes from radiation-induced DNA fragmentation.

Figure 3 shows the effect of varying concentrations of WR-1065 on DNA fragmentation in thymocytes after different doses of γ-irradiation. The inhibition of radiation-induced DNA fragmentation depended on the concentration of WR-1065 during postirradiation incubation. At 2.5 mM WR-1065 there was 1 to 10% DNA fragmentation in thymocytes exposed to 1.5–6 Gy γ-radiation, and maximum inhibition of DNA fragmentation was obtained at 5 to 10 mM WR-1065. We used 10 mM WR-1065 in all our studies, and it was not toxic to the cells, as shown in Figure 4. Cell viability was assessed by trypan blue dye exclusion method. After irradiation the fraction of dead cells increased progressively with time. Addition of WR-1065 to unirradiated or irradiated thymocytes maintained their viability at 90 to 95% (Fig. 4). The results also indicate that DNA fragmentation in irradiated thymocytes precedes the loss of viability (Figs. 1 and 4), which is consistent with the results reported by others (1–6).

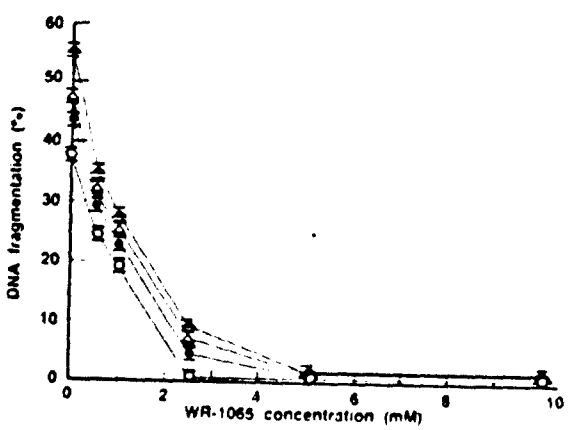

Figure 3. Effect of increasing concentrations of WR-1065 on radiation-induced DNA fragmentation in thymocytes. Thymocytes were exposed to different doses of γ-radiation, and percentage of DNA fragmentation was measured after 8 h incubation with increasing concentrations of WR-1065. The results are mean ± SE from three experiments. ○, 1.5 Gy; ●, 3.0 Gy; △, 4.5 Gy; ▲, 6.0 Gy.

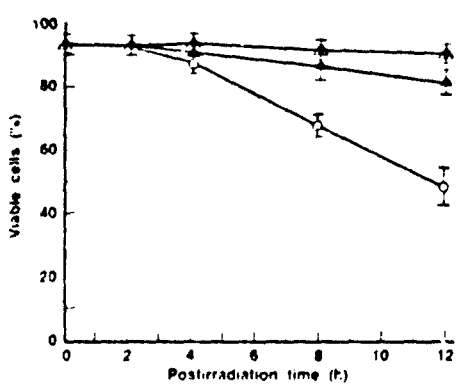

Figure 4. Effect of 10 mM WR-1065 on cell death at various times after irradiation at 6.0 Gy. Viability is expressed as the percentage of cells that excluded trypan blue. The results are mean ± SE from three experiments. ▲, unirradiated; △, unirradiated + WR-1065; ○, irradiated; ●, irradiated + WR-1065.

In the above-mentioned studies, thymocytes were incubated with WR-1065 after irradiation. Further studies were carried out to determine whether the addition of WR-1065 before irradiation protects the thymocytes from DNA fragmentation. Thymocytes were exposed to γ-radiation after 60 min incubation with 10 mM WR-1065. After irradiation, the cells were centrifuged, resuspended in fresh medium without WR-1065, and DNA fragmentation was measured after 8 h of postirradiation incubation. The results shown in Table I indicate that WR-1065 added to thymocytes before irradiation does not protect them from radiation-induced DNA fragmentation.

WR-1065 blocks dexamethazone- and calcium ionophore A23187-induced DNA fragmentation in thymocytes. Glucocorticoid hormones and calcium ionophores are known to stimulate apoptosis in thymocytes, which involves extensive DNA fragmentation by Ca^{2+}- and Mg^{2+}-dependent nuclear endonuclease (4–6). We studied the effect of WR-1065 on dexamethazone-induced and calcium ionophore A23187-stimulated DNA fragmentation in thymocytes. Thymocytes were incubated with increasing concentrations of either dexamethazone or calcium ionophore A23187 with and without WR-1065 in medium at 37°C for 8 h. Dexamethazone and calcium ionophore A23187 stimulated concentration-dependent DNA fragmentation and cell death (Table II) in thymocytes. WR-1065 blocked the dexamethazone-induced and calcium ionophore-stimulated DNA fragmentation and cell death (Table II) in thymocytes.

WR-1065 inhibits Ca^{2+}- and Mg^{2+}-dependent DNA fragmentation in thymocyte nuclei. Several studies indicate that DNA fragmentation observed during apoptosis

TABLE I

Effect of preirradiation incubation of WR-1065 on DNA fragmentation in thymocytes

Treatment	DNA Fragmentation (%)		
	0 Gy	3.0 Gy	6.0 Gy
Control	15.8 ± 0.5[a]	43.7 ± 1.1	50.5 ± 0.8
WR-1065[b]	14.3 ± 1.5	40.5 ± 3.2	49.7 ± 1.7

[a] The results are mean ± SE from three experiments.

[b] Thymocytes were irradiated after 60 min incubation with 10 mM WR-1065 in TCM containing 100 U/ml catalase. After irradiation, cells were centrifuged, resuspended in fresh medium without WR-1065, and incubated for 8 h. Incubations and DNA analysis were carried out under the conditions mentioned in *Materials and Methods*.

TABLE II

Effect of WR-1065 on cell viability and DNA fragmentation in thymocytes exposed to dexamethazone or calcium ionophore A23187

Treatments	Viability (%)[a]		DNA Fragmentation (%)[b]	
	− WR-1065	+ WR-1065	− WR-1065	+ WR-1065
Dexamethazone				
0 nM	95 ± 3	96 ± 2	12.5 ± 1.7	1.5 ± 0.6
100 nM	78 ± 6	91 ± 3	40.0 ± 2.5	1.8 ± 1.2
200 nM	69 ± 10	92 ± 5	47.2 ± 1.2	2.1 ± 1.3
1 μM	40 ± 4	90 ± 4	53.1 ± 1.5	1.2 ± 0.9
10 μM	25 ± 8	91 ± 6	57.5 ± 1.2	2.9 ± 1.2
A23187				
0 nM	95 ± 3	96 ± 2	14.5 ± 1.6	1.0 ± 0.5
100 nM	80 ± 7	91 ± 3	20.6 ± 1.8	1.0 ± 0.5
200 nM	75 ± 5	89 ± 4	26.1 ± 0.9	1.7 ± 0.7
400 nM	67 ± 6	90 ± 2	33.6 ± 1.8	1.9 ± 0.9
1 μM	56 ± 7	87 ± 6	44.1 ± 0.7	1.9 ± 0.8

[a] Viability is expressed as the percentage of cells that excluded trypan blue after 8 h incubation with the indicated concentrations of either dexamethazone or calcium ionophore A23187 with or without WR-1065.

[b] DNA fragmentation was determined after 8 h incubation. The results are mean ± SE from three experiments.

of thymocytes is due to action of a Ca^{2+}- and Mg^{2+}-dependent nuclear endonuclease that cleaves host chromatin into oligonucleosome-length fragments (1–6). We tested the effect of WR-1065 on Ca^{2+}- and Mg^{2+}-dependent DNA fragmentation in thymocyte nuclei. Nuclei were isolated from thymocytes and incubated in Tris-buffered (10 mM, pH 7.5) isotonic sodium chloride with and without added Ca^{2+} and Mg^{2+}. We found that most of the DNA remained intact in thymocyte nuclei incubated with Mg^{2+} or Ca^{2+} alone, but when both ions were present about 68% of DNA was fragmented (Table III). Interestingly, WR-1065 inhibited Ca^{2+}- and Mg^{2+}-dependent DNA fragmentation in thymocyte nuclei (Table III). The results of this study suggest that WR-1065 could be protecting the thymocytes from DNA fragmentation by inhibiting the action of Ca^{2+}- and Mg^{2+}-dependent nuclear endonuclease.

DISCUSSION

The results of these studies clearly indicate that WR-1065 inhibits the internucleosomal DNA fragmentation and cell death in thymocytes exposed to γ-radiation, dexamethazone, and calcium ionophore A23187. WR-1065 is known to protect mammalian cells from radiation-induced reproductive death when incubated with the cells before and during irradiation; it does not inhibit reproductive death when added to cells after irradiation (22–25, 30–32). When mammalian cells are exposed to ionizing radiation, DNA damage occurs during irradiation due to direct interaction of free radicals with DNA and it can be measured immediately after irradiation (33). It is thought that the presence of WR-1065 in cells during irradiation prevents reproductive death by interferring with the interactions of radiation-induced free radicals with DNA (30–32).

The ability of WR-1065 to protect against free radical interaction with DNA is apparently not related to its ability to prevent apoptosis in irradiated cells. DNA fragmentation, a characteristic of apoptosis, is unaffected by incubating cells with WR-1065 before irradiation; fragmentation is at the same level in the irradiated cells pretreated with WR-1065 as it is in cells not pretreated with WR-1065 (Table I). In thymocytes there is no DNA fragmentation immediately after irradiation; fragmentation begins at 2 to 3 h postirradiation and increases with time (Fig. 1). In apoptosis, DNA fragmentation appears to be a distinctively postirradiation cellular process. Inasmuch as the presence of WR-1065 in the cell after irradiation completely blocks DNA fragmentation, it may be inhibiting a postirradiation cellular process responsible for DNA fragmentation. It has been reported that the magnitude of radioprotection against reproductive death depends on the intracellular concentration WR-1065 at the time of irradiation (32). The ability of WR-1065 to protect against DNA fragmentation during apoptosis depends on the concentration of WR-1065 during postirradiation incubation (Fig. 3).

TABLE III

Effect of WR-1065 on activation of endogenous endonuclease in isolated thymocyte nuclei.[a]

Treatments	$CaCl_2$, 5 mM	$MgCl_2$, 10 mM	DNA fragmentation (%)
Nuclei	−	−	0.7 ± 0.4
	−	+	0.8 ± 0.1
	+	−	25.0 ± 1.6
	+	+	68.2 ± 1.3
Nuclei + WR-1065[b]	−	−	0.3 ± 0.3
	−	+	0.6 ± 0.1
	+	−	0.5 ± 0.1
	+	+	0.4 ± 0.1

[a] Thymocyte nuclei were incubated with different concentrations of cations at 37°C for 4 h and DNA was estimated as mentioned in *Materials and Methods*. −, without; +, with.

[b] 10 mM WR-1065. The results are mean ± SE from three experiments.

Dexamethazone and calcium ionophore A23187 induce a similar degree of DNA fragmentation in thymocytes as those observed after irradiation. WR-1065 inhibits DNA fragmentation in these cases also, suggesting a common mechanism of action. Our studies with thymocyte nuclei indicate that WR-1065 inhibits a Ca^{2+}- and Mg^{2+}-dependent process responsible for DNA degradation. In our studies, more than 50% of the DNA was fragmented in isolated nuclei incubated in the presence of Ca^{2+}- and Mg^{2+} (Table III). DNA isolated from irradiated cells (Fig. 2, *lane D*) and cation-treated unirradiated nuclei (not shown) showed no difference in their electrophoretic patterns. The specific pattern of degradation of DNA into oligonucleosomal subunits suggests that an endonuclease may be involved in the process. It is possible that a Ca^{2+}- and Mg^{2+}-dependent nuclease may be constitutively present in an inactive form in thymocyte nuclei. When optimum concentrations of Ca^{2+} and Mg^{2+} are present, the enzyme may be activated to degrade DNA into oligonucleosomal subunits. A nuclease of similar specificity has been described in nuclei of thymocytes and other mammalian cells (34–37). Nuclei incubated with WR-1065 showed no cation-dependent DNA fragmentation (Table III), suggesting the inhibition of action of nuclease.

A variety of molecular and cellular mechanisms has been proposed to explain the ability of WR-1065 to protect mammalian cells from radiation-induced reproductive death (20, 38). The mechanism of protection offered by WR-1065 in our experiments is not clear. However, several possible mechanisms for the action of WR-1065 in thymocyte apoptosis may be suggested from the above results. First, WR-1065 may inhibit the DNA degradation by altering the structure of internucleosomal region in chromatin. WR-1065 binds to DNA and nuclear proteins in mammalian cells (39). This binding in thymocyte nuclei may alter the conformation of chromatin in such a way that internucleosomal region may not be available for degradation of chromatin into oligonucleosomal subunits. Second, WR-1065 may inactivate the enzyme responsible for DNA degradation. WR-1065 forms mixed disulfides with sulfhydryl groups in protein (20, 38). It is possible that WR-1065 may inactivate the nuclear endonuclease by forming mixed disulfides with sulfhydryl groups of the enzyme. Third, WR-1065 may regulate the cellular transport of cations necessary for DNA degradation. Studies indicate that WR-2721 and WR-1065 modulate calcium metabolism in chronic renal failure (40) and in hypercalcemia of malignancy (41). Recently, WR-1065 was shown to prevent calcium entry and cell death in U937 human premonocytic cell line exposed to hydrogen peroxide (42). The precise cellular mechanism by which WR-1065 regulates calcium transport is unknown. WR-1065 does not form chelation complexes with Ca^{2+} and Mg^{2+} (43). It may either act directly on calcium channels in the membrane or inhibit lipid peroxidation of membranes and prevent calcium entry. Lipid peroxidation alters membrane permeability and increases calcium influx, and it can be induced by several oxidants includ-

ing ionizing radiation (44–46). WR-1065 is known to inhibit the lipid peroxidation of membranes (47, 48). Studies are in progress to understand the precise cellular and molecular mechanism of action of WR-1065 in protecting thymocytes from apoptosis.

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